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Data on molecular taxonomy and genetic diversification of the European Hermit beetles, a species complex of endangered insects (Coleoptera: Scarabaeidae, Cetoniinae, *Osmoderma*)

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Abstract

A molecular analysis was carried out on the European hermit beetles (the *Osmoderma eremita* species complex) to explore their genetic diversification and the robustness of previous morphologically based taxonomic arrangements. Complete sequences of mtDNA cytochrome C oxidase I gene were obtained from 26 individuals. Mean levels of interspecific sequence divergence ranged from 0.044 to 0.186. The results indicate a clear-cut distinction between two clades. The first one includes the W-European *O. eremita* Scopoli 1763; and the two Italian endemic taxa *Osmoderma italicum* Sparacio, 2000 and *Osmoderma cristinae* Sparacio, 1994; from southern peninsular Italy and Sicily, respectively. The second one includes the widespread E-European *Osmoderma barnabita*; and the southern Balcanic *Osmoderma lassallei* Baraud and Tauzin, 1991 from Greece and European Turkey. Within the two clades, molecular and morphological data well support a specific rank for *O. lassallei* and *O. barnabita* on one side, and for *O. eremita* and *O. cristinae* on the other side, while the taxonomic position of *O. italicum*, more closely related to *O. eremita*, is still uncertain. Current geographical distribution, interspecific genetic diversification, and very low levels of intraspecific genetic divergence in western European populations of *O. eremita* sensu stricto are hypothesized to be the result of multiple speciation events (mainly occurred in refugial forest areas of the Italian and Balkan peninsulas and Sicily before and during the Pleistocene glacial peaks), followed by fast post-glacial northward and westward expansion of some species.

Key words: cytochrome C oxidase I gene – molecular taxonomy – sibling species – threatened species – *Osmoderma* – Coleoptera

Introduction

Hermit beetles are a group of flower chafers (Scarabaeidae: subfamily Cetoniinae: tribe Trichiini) of the genus *Osmoderma* LePeletier de Saint-Fargeau and Serville, 1828. They are large scarab beetles (more than 30 mm long) that live in old hollow trees. We adopt here the current generic name *Osmoderma* instead of the recently re-introduced name *Gymnodus* Kirby, 1827 (Ádám 1994, 2003; Gusakov 2002), according to a submitted proposal to the International Commission on Zoological Nomenclature (ICZN) and its recent opinion (Krell et al. 2006; Audisio et al. 2007; Barclay 2007; ICZN 2007).

This genus includes a dozen of species widely spread throughout the Palaearctic and the Nearctic regions (see the world checklist in Audisio et al. 2007). The geographic distribution and the ecological traits of the European species, joined together under the name *Osmoderma eremita* sensu lato, have been recently summarized by Ranius and Nilsson (1997), Schaffrath (2003a,b), Ranius (2000, 2001), Ranius and Hedin (2001, 2004) and Ranius et al. (2005). These studies, supported by many other surveys conducted at local level, evidenced a strong decline suffered by this taxonomic group throughout its distribution range, and reported extinction from some countries owing to habitat loss and intensive forest management. For this reason, *O. eremita* has been listed as a priority species in Annex IV of the EU's Habitat Directive (Luce 1996, 2001; Galante and Verdú 2000; Audisio et al. 2003; Ranius et al. 2005).

As discussed in a series of recent contributions (Sparacio 1994, 2000; Tauzin 1994a,b, 1996, 2000, 2002; Krell 1997; Gusakov 2002; Audisio et al. 2003; Dutto 2003; Ranius and Hedin 2004, 2005), under the name *O. eremita* are probably included at least two or more distinct species or semi-species, whose taxonomic rank has been a matter of strongly controversial interpretations. In fact, some studies (Nüssler 1986; Sparacio 1994, 2000; Tauzin 1994b, 2006; Gusakov 2002; Audisio et al. 2003; Ranius and Hedin 2004; Brustel 2004) suggested a morphological distinction of at least two up to five substantially allopatric semi-species, whose actual taxonomic position is difficult to ascertain:

- *Osmoderma eremita* Scopoli, 1763, widespread in western Europe, eastwards to Germany and western Slovenia.
- *Osmoderma cristinae* Sparacio, 1994, confined to Sicily.
- *Osmoderma italicum* Sparacio, 2000, occurring in southern Italy.
- *Osmoderma lassallei* Baraud and Tauzin, 1991, distributed in Greece and European Turkey.
- *Osmoderma coriarium* De Geer, 1774; *sensu* Gusakov, 2002; from eastern Europe (this taxon hereafter is treated under the combination *Osmoderma barnabita* see later and in the Commented World Checklist of Audisio et al. 2007).

However, until very recently, *O. eremita* s.l. was provisionally treated as one species with clear geographic morphological variation and three recognized distinct subspecies (*O. eremita eremita*, *O. eremita lassallei* and *O. eremita cristinae* (Krell 1997, 2004; Shokhin and Bozadjiev 2003).

The aim of this paper is mainly to test the genetic diversification among the European species or semi-species of the *O. eremita* complex; to achieve data towards a reasonable and objectively supported taxonomic arrangement for the whole complex; and to provide a preliminary scenario of their phylogenetic relationships based on molecular data. Audisio et al. (2007) can be referred for the rationale of the specific epithets used in the present study.

Materials and Methods

Sampling

The analysed specimens were collected from localities listed in Table 1. Dying, but still alive adults (in four cases previously attacked and mutilated by birds) were collected at the end of their reproductive period, and directly preserved in tubes containing pure acetone for analysis (Fukatsu 1999). Species identification was carried out in laboratory using morphological characters (Tauzin 1994a; b; Sparacio 2000; Gusakov 2002; Dutto pers. comm. 2007).

DNA extraction, PCR and sequencing

The total genomic DNA was isolated using standard phenol/chloroform protocol (Sambrook and Russell 2000). Metafemoral muscles were dissected from each specimen, dried at 37°C in 1.5 ml microcentrifuge tubes, and after the addition of 5 µl of a solution of proteinase K (20 mg ml^{-1}) in 200 µl of lysis-digestion buffer (EDTA 10 mM, Tris 100 mM, pH 7.5, NaCl 300 mM, 2% SDS), homogenized on ice using a pestle. The cell lysis solution was incubated at 65°C for at least 3 h. The mixture was treated with phenol, phenol-chloroform (1 : 1) and chloroform, and the DNA was precipitated in two volumes of 95% EtOH and one-tenth volume of 3 M sodium acetate. The DNA was then pelleted, washed once with 80% EtOH, and resuspended in 50 µl of TE (Tris-Ethylenediamine tetracetic acid buffered solution) buffer 1× pH 7.5.

Polymerase chain reactions were performed in order to amplify the whole mitochondrial cytochrome C oxidase subunit I (COI) gene. The amplifications by means of universal primers, such as those published by Simon et al. (1994), were unsuccessful, and in order to amplify the whole target gene, we set out specific primers [C1-OSMO-1358(+) and C1-OSMO-3086(−)]; a modification of the forward primer was used to

Table 1. Taxa and specimens examined for molecular analyses[1]

Species/acronym	Locality	Date of collection	Accession # COI
Osmoderma eremita			
OE1.1	Italy, Lazio, Rome, Villa Borghese Park	18.vii.2001	AJ880680
OE2.1	Sweden, Skåne province, Hallands Vaderö Island	01.vii.2002	AJ880681
OE2.2	Sweden, Skåne province, Hallands Vaderö Island	01.vii.2002	AJ880682
OE3.1	Italy, Lombardia, Sondrio province, Tovo di S. Agata	20.vii.2003	AJ880683
OE5.1	France, Orne, Coulmer, Les Portes	12.vii.2005	AM412372
OE6.1	France, Sarthe, Mayet, Les Blottes	05.viii.2005	AM412373
OE6.2	France, Sarthe, Mayet, Les Blottes	05.viii.2005	AM412374
OE7.1	France, Pyrénées-Atlantiques, Sare, Forêt de Sare	20.vi.2005	AM412375
OE7.2	France, Pyrénées-Atlantiques, Sare, Forêt de Sare	20.vi.2005	AM412376
OE7.4	France, Pyrénées-Atlantiques, Sare, Forêt de Sare	20.vii.2005	AM412377
OE8.1	Italy, Lombardia, Sondrio province, Grosotto	27.viii.2005	AM423158
OE9.1	Slovenia, 12 Km south-east of Kranj	20.viii.2006	AM423159
OE14.1	Germany, Hessen, Staatspark Karlsaue near Kassel	vii.2006	AM423156
OE15.1	Germany, Bavaria, Rothenbuch, Eichhal	vii.2006	AM423157
Osmoderma barnabita			
OB1.1	Croatia, Plitvice Lakes National Park	30.vii.2002	AJ880684
OB2.1	Greece, Ioannina province, Katara pass (Pindus Mts.)	28.viii.2005	AM412378
OB3.2	Germany, Saxony, near Weisswasser, Hagberg	12.vi.2005	AM412379
OB4.1	Slovakia, Zvolen, Dobrà Niva	vii.2006	AM423160
Osmoderma lassallei			
OL1.1	Greece, Larissa province, Mount Ossa, Kokkino-Nero	30.vii.2003	AJ880685
OL2.1	Greece, Larissa province, Omolion	27.vii.2005	AM412380
OL2.2	Greece, Larissa province, Omolion	12.vii.2005	AM412381
OL3.1	Greece, Larissa province, Mount Ossa, Spilea	27.vii.2005	AM423161
Osmoderma italicum			
OI1.1	Italy, Basilicata, Potenza province, Terranova di Pollino	17.vii.2003	AJ880686
Osmoderma cristinae			
OC1.1	Italy, Sicily, Palermo province, Madonie Mts., Gibilmanna	12.vii.2003	AJ880679
OC2.1	Italy, Sicily, Messina province, Nebrodi Mts., Muto between Casa Cicalda and Casa Forestale above San Fratello	06.vii.2005	AM412382
OC2.3	Italy, Sicily, Messina province, Nebrodi Mts., Muto between Casa Cicalda and Casa Forestale above San Fratello	20.vii.2005	AM412383

COI, cytochrome C oxidase I gene.
[1]For the specimens OE1.1, OE2.1, OE2.2, OE3.1, OB1.1, OC1.1, OL1.1 and OI1.1, the complete COII sequences were submitted to European Molecular Biology Laboratory databank, with the following accession numbers: AJ884601, AJ884602, AJ884603, AJ884604, AJ884605, AJ884606, AJ884607 and AJ884608.

Table 2. Oligonucleotide primers used during PCR amplifications and sequencing reactions

Primer	Sequence (5′-3′)	Reference
C1-OSMO-1358(+)	TTA TCT TTA AGC CTT AGG GAT C	Present study
C1-OSMO-1358las(+)	TTA TCT TTA AGC CTT AGA GAT T	Present study
C1-OSMO-3086(−)	GGC GGA ATT TCA GGT TGC	Present study
C1-OSMO-SQ-1930(+)	GAG CAT CAG TAG ATT TAG C	Present study
C1-OSMO-SQ-1930las(+)	GAG CAT CAG TAG ACT TGG C	Present study
C1-OSMO-SQ-1961las(+)	TCA TCT AGC CGG AAT TTC	Present study
C1-OSMO-SQ-1961bar(+)	TCA TCT AGC AGG AAT TTC	Present study
C1-OSMO-SQ-3020(−)	GCA CTA ATC TGC CAT ATT	Present study

Numbers refer to the position of the primer 3′-end mapped on the *Drosophila yakuba* complete mitochondrial genome (Clary and Wolstenholme 1985).

amplify the *O. lassallei* and *O. barnabita* samples [C1-OSMO-1358-las(+); see Table 2]. The PCR strategies were set up also with the target in mind of optimizing the next sequencing step that indeed were easily derived from the former ones. The COI gene was completely sequenced using three reactions employing the two amplification primers [C1-OSMO-1358(+) and C1-OSMO-3086(−) for *O. eremita*, *O. italicum* and *O. cristinae* samples; C1-OSMO-1358las(+) and C1-OSMO-3086(−) for *O. lassallei* and *O. barnabita* samples] along with a third primer specifically designed in a middle position of the region [C1-OSMO-SQ-1930(+) for *O. eremita*, *O. italicum* and *O. cristinae* samples; C1-OSMO-SQ-1930las(+), C1-OSMO-SQ-1961las(+), C1-OSMO-SQ-1961bar(+) for *O. lassallei* and *O. barnabita* samples] or, in the 3′ terminal position, C1-OSMO-SQ-3020(−) only for *O. lassallei* and *O. barnabita* samples.

Polymerase chain reactions were performed on a Perkin Elmer GeneAmp PCR System 2400 thermal cycler with the following amplification conditions: initial denaturation at 95°C for 5 min followed by 33 cycles of 1 min each at 94°C, 30 s annealing at 55°C, 1 min extension at 72°C and a subsequent 7 min elongation step at 72°C. Reactions were performed in a 50 µl volume containing $(NH_4)_2SO_4$ 16 mM, Tris-HCl 67 mM (pH 8.8 at 25°C), $MgCl_2$ 3 mM, Tween-20 0.01%, 1 mM of each deoxynucleotide, 0.8 pmol of each primer, 1.25 units of Taq DNA polymerase (Fisher Molecular Biology, Hampton, NH, USA). The amplified products were purified by Exo-SAP enzymatic reaction or by ultra-centrifugation on silica filter columns with the NucleoSpin Extract Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The fragments were then sequenced at the BMR-genomics S.P.A. (Padua, Italy), employing an Applied Biosystem 3100 Genetic Analyzer and using a Dye Terminator Ready Reaction Kit (Perkin Elmer, Waltham, MA, USA) according to the manufacturer's protocol.

Sequences and phylogenetic analyses

The COI sequences were edited and aligned using the Staden Package software program ver. 2003.1.6 (Bonfield et al. 1999–2002; Staden et al. 2003). All gap sites were deleted during subsequent analyses (see later), therefore based on 1545 positions. Sequence analyses were conducted using MEGA version 3.1 (Kumar et al. 2004). The codon position and the open reading frame (ORF) assignation were inferred by comparison with those reported in Lunt et al. (1996). The sequences were translated using the *Drosophila* mitochondrial genetic code (De Bruijn 1983; Clary and Wolstenholme 1985).

Best fit of molecular evolution model to our data was performed using TREEFINDER ver. January 2008 (http://www.treefinder.de/; Jobb et al. 2004, 2008): the program first reconstructs a preliminary maximum likelihood (ML) tree from the sequences under a simple model. Then, it computes likelihood values for the preliminary tree under each of a set of candidate models, along with edge lengths and model parameters. Finally, the program returns the best-fit model based on the Akaike's information criterion (Jobb et al. 2004, 2008).

A general time reversible model + γ distribution (see Table S1 for detailed parameters) was set as a substitution model to perform a maximum likelihood (ML) phylogeny reconstruction by means of the same software. Distance matrix was computed following the substitution model selected by TREEFINDER. Phylogenetic relationships were also performed using PAUP* 4.0b10 for MS-Windows (Swofford 2002) by the Neighbor-joining algorithm (NJ; Saitou and Nei 1987) and the maximum parsimony criterion (MP; Fitch 1971). The parsimony analysis was carried out without any weighting scheme, using a heuristic search and with no selected outgroup. Node supports were evaluated by running 1000 replicates of bootstrap re-sampling for each phylogenetic analysis (ML, MP, NJ) (Felsenstein 1985).

Results

Nucleotide composition

For all but OE14.1 and OE15.1 individuals belonging to the analysed taxa, COI sequences of 1551 bp were submitted to EMBL databank, and their accession numbers are listed in Table 1. The two aforementioned individuals exhibit a shorter sequence (1545 bp) because of a six-site gap in their sequences (positions 612–617). Comparing the aligned sequences with that of *Drosophila yakuba* (Clary and Wolstenholme 1985; see also Lunt et al. 1996), the putative initiation codon seems to be TCA for all studied taxa, whose shared ORF resulted in a sequence of 517 amino acids. Table 3 summarizes the results of the nucleotide and amino acid composition analysis for the target gene.

As expected for the insect mtDNA (Simon et al. 1994), the marker shows a bias in the A+T base composition (64.5%, $C = 0.193$; see Table 3 for the definition of the C parameter). The bias is not equally distributed over the three codon positions, certainly owing to the high nucleotide substitution rate at the third position ($C = 0.379$). The Ti/Tv ratios, averaged over all taxa, show a very high frequency of the transitions, strongly suggesting a pattern of substitution not yet saturated. Two hundred and sixty out of 1545 sites were variable (16.8%). As expected, the greatest amount of variation is found at the third codon position (233/260, equal to 89.6% of the total variation). The pattern of the amino acid substitution, with a very low level of variation (2.5%), is likely owing to functional constraints for the polypeptide chain and to the scored low levels of nucleotide substitution in first and second positions (Table 3).

Sequence divergence and phylogenetic analyses

Tables 4 and S1 show the scored values of the genetic distances among all sampled individuals (Table S1) and taxa (Table 4) computed using the substitution model selected by TREEFINDER. The pairwise values ranged from 0.000 (OE6.1 versus OE6.2) to 0.218 (OL2.2 versus OE15.1). Among the putative species, the average values ranged from 0.044 (*O. eremita* versus *O. italicum*) to 0.186 (*O. eremita* versus *O. lassallei*).

Fig. 1 displays the consensus topology (Jobb 2008) obtained by means of the ML method. The numbers at nodes represent the bootstrap values after 1000 replicates, while boxed numbers next to the branches refer to the averaged edge lengths (Jobb 2008). Both the MP and the NJ consensus trees gave out a topology identical to the ML one, and therefore, it

Table 3. Nucleotide and amino acid composition and variation for the cytochrome C oxidase I gene

	n.n.	A	T	C	G	A+T	Bias	Ti/Tv	v.s.	Pi	v.a.a.
Total	1545	30.2	34.3	20.3	15.1	64.5	0.193	4.2	260	208	13/515
1st position	515	30.0	26.2	18.4	25.4	56.2	0.088	4.0	22	–	–
2nd position	515	17.5	41.4	25.2	16.0	58.9	0.220	nc	5	–	–
3rd position	515	43.1	35.5	17.3	4.1	78.5	0.379	4.3	233	–	–

n.n., nucleotide number; v.s., variable sites; Pi, parsimony informative sites; v.a.a., variable amino acids.
The base composition bias was computed using the formula: $C = 2/3 \sum |c_i - 0.25|$, where c_i is the frequency of the ith base (Funk 1999).

Table 4. Averaged interspecific and intraspecific divergences for cytochrome C oxidase I gene. Values of intraspecific variability are listed in the second column

		O. barnabita	O. cristinae	O. eremita	O. italicum
O. barnabita	0.018				
O. cristinae	0.017	0.157			
O. eremita	0.016	0.179	0.068		
O. italicum	n.c.	0.171	0.060	0.044	
O. lassallei	0.007	0.085	0.163	0.186	0.178

O., *Osmoderma*.

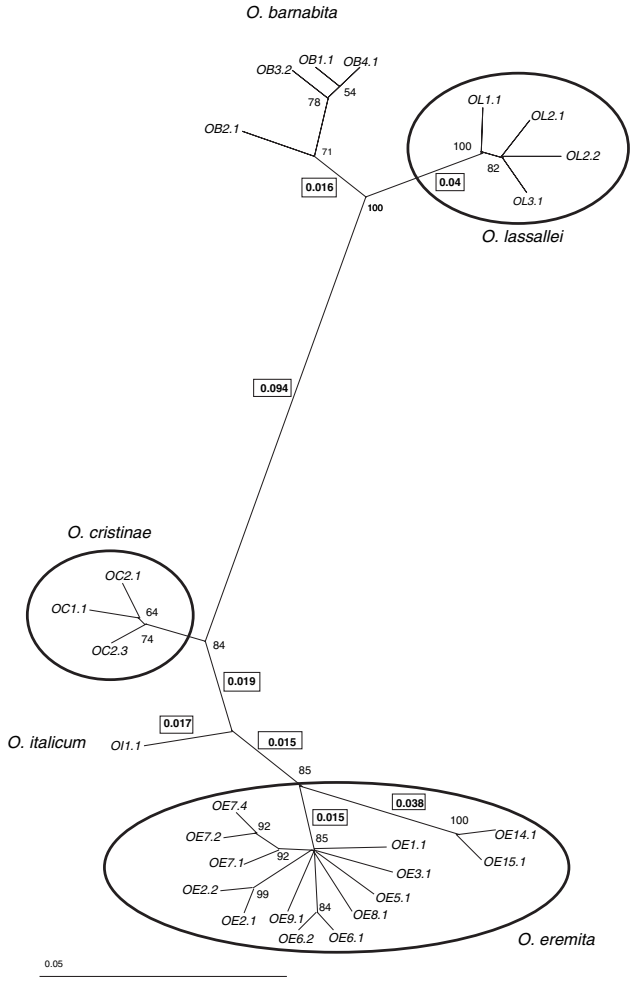

Fig. 1. Unrooted consensus of Maximum Likelihood trees computed on the complete COI dataset; the numbers at nodes refer to bootstrap values after 1000 replicates, while boxed numbers next to the branch represent the averaged edge lengths (scale bar refer to averaged edge lengths)

was chosen not to report them in the present paper. Parsimony analyses produced 100 trees all equal in length (tree length = 405; consistency index = 0.68; retention index = 0.90, rescaled consistency index = 0.65) that show the same topology of the main clusters.

It is worth noting that in all phylogenetic reconstructions, individuals are placed in monophyletic clades according to their previously assigned morphologically based taxonomic status. All these clusterings are robust, as clearly depicted by the high bootstrap values recovered, notably in the ML topology. Gained topology is depicted (Fig. 1) as unrooted tree because all analyses carried out by using outgroup taxa, among those available in the EMBL databank [i.e. *Protaetia cuprea* (Fabricius 1775) from Europe; Cetoniinae, Cetoniini] or newly sequenced for the aims of this paper [*Aethiessa floralis* (Fabricius, 1787) from Sicily; Cetoniinae, Cetoniini], resulted in no more informative topologies of the evolutionary relationships among the studied taxa. In other words, adding, for instance, the COI sequence of *Aethiessa floralis* or of *P. cuprea*, the gained relationships among the *Osmoderma* studied entities were unchanged when compared with the unrooted topology, in both cases showing an unresolved basal politomy. In fact, both taxa showed a very marked divergence with respect to all species included in the *O. eremita* species complex, likely owing to saturation of the target marker at higher taxonomic levels. For this reason, presenting the unrooted topology of the *Osmoderma* taxa alone is preferred.

Discussion

The ML, MP and NJ analyses well support the distinction of two clusters grouping *O. barnabita* and *O. lassallei* on one side, and *O. eremita*, *O. italicum* and *O. cristinae* on the other side (Table 4; Fig. 1). The average level of genetic divergence between the two identified groups is equal to 0.179. All trees show a good phylogenetic cohesion of the studied taxa along with their grouping in monophyletic groups and their reciprocal distinction. These interspecific relationships are mirrored in the scored genetic distance values showed in Table 4. These figures are truly comparable with values recently scored in other scarab beetles (Coleoptera, Scarabaeidae) for taxa currently interpreted as related but separated species (Villalba et al. 2002; Micó et al. 2003; Cabrero-Sañudo and Zardoya 2004; Tassi et al. 2004), or well-distinct sub-species (Carisio et al. 2004). Within the group clustering *O. eremita*, *O. italicum* and *O. cristinae*, the values outline a more strict degree of genetic relatedness between *O. eremita* and *O. italicum* (Table 4), suggesting a possible interpretation of the latter as subspecies or only significant geographic form of the widespread *O. eremita* s.s., and is still to be investigated in the near future. Similar pattern of relationships occurs between the group OE14.1 + OE15.1 and all the western European populations analysed. Indeed, the cited individuals come from the eastern portion of the *O. eremita* current distribution range (Central Eastern Germany), exhibit a relatively higher level of genetic divergence, and bear rather different haplotypes owing to the deletion of six sites at 612–617 positions. On the contrary, the values of genetic divergence between *O. cristinae* and both *O. eremita* or *O. italicum* (Table 4), combined with the morphological differentiation (Sparacio 2000) and the isolated geographic range (Fig. 4) of *O. cristinae*, seem to better support a specific rank of the Sicilian group of populations. Finally, the intra-specific values of genetic variation (Table 4) suggest a good degree of genetic cohesion within the western European populations of *O. eremita*, probably owing to a very recent (post-Würmian) origin of its Scandinavian populations, which followed the northward colonization of woodlands from south west Europe, after the last Ice Age (Taberlet et al. 1998; Hewitt 1999).

Taking into account evidence from a huge number of studies, a 2% divergence between pairs of COI sequences of related species is going to be considered the threshold level

between subspecies and species ranks (Hebert et al. 2003a; b). In fact, more than 98% of well-defined species pairs examined by Hebert et al. (2003a,b) showed values of divergence higher than 2%, while intraspecific divergence was rarely more than 2%, and almost always less than 1% (Avise 2000). However, it is a well-known fact that nucleotide substitution rates might greatly vary among different organisms, even among the same genes, or among different domains of a gene within the same organism (Caccone and Sbordoni 2001), and caution should be adopted in evaluating scored genetic divergence for taxonomic purpose. It is then strongly advised not to regard the threshold level between subspecies and species ranks proposed by Hebert et al. (2003a,b) as a dogma, as exemplified by our results (Tables 4 and S1).

In order to tentatively date the splitting of the studied taxa, we estimated the divergence times among them (Nei 1987) assuming a mtDNA mutation rate of 2% per million years (MY) (Stireman et al. 2005). Under this assumption, the divergence time estimated between *O. barnabita* s.l. (= *O. barnabita* + *O. lassallei*) and *O. eremita* s.l. (= *O. eremita* + *O. cristinae* + *O. italicum*) would date around 8–9 MY. The divergence among *O. barnabita* and *O. lassallei* would date around 2 MY, and a similar timing could be estimated for the splitting of *O. cristinae* from the remaining taxa included in *O. eremita* s.l. Instead, the observed average divergence among *O. eremita* s.str. and *O. italicum* is comparable with the already discussed high intraspecific genetic variability of *O. eremita*, mainly attributed to the isolated genetic position of the studied German specimens. Therefore, the use of additional molecular markers and a wider sampling of *O. eremita* in eastern Germany and of '*O. italicum*' in southern Italy are needed before any conclusions and time estimations could be drawn. However, on the basis of the available data, we hypothesize that the differentiation of *Osmoderma* populations (following the geographic isolation of mesophilous woodlands) is likely dated before most of the late Pliocene/Pleistocene glaciations. The climatic cooling probably confined *Osmoderma* populations several times to some scattered refuge areas of the Balkan and Italian peninsulas, and Sicily, causing further fragmentation of their genetic pools into more or less isolated groups. In this way, the ancestral European *Osmoderma* might have been scattered in isolated southern refugia for many thousands of years during the Ice Ages and then expanded northwards following climate amelioration (Taberlet et al. 1998; Hewitt 1999; Vogel et al. 1999).

Conclusions

The divergence levels between European *Osmoderma* species, assessed by the present study, should be calibrated with data gathered from closely related but neatly distinct taxa (e.g. the even rarer species of the related genus *Gnorimus* LePeletier de Saint-Fargeau and Serville, 1828, or less closely related *Osmoderma* species from the Caucasus, the Palaearctic Far East or the Nearctic Region; see Audisio et al. 2007). Genetic data on hermit beetles should be increased both in terms of sampled specimens and examined populations in order to assess the specific or subspecific rank of all the taxa assigned to *Osmoderma*. But the rarity of most hermit beetle taxa and the associated legal constraints in collecting them are a serious and obvious drawback for taxonomic researches on this genus. Taking all these considerations into account, the following provisional taxonomic conclusions and faunistic implications (the taxonomic framework supported by molecular evidences obtained in this research is extensively discussed in Audisio et al. 2007) are introduced here.

- The *O. eremita* complex includes in Europe at least two distinct clades, the first one (*O. eremita* s.l.), comprising taxa distributed in western Europe (Fig. 2: northern Spain, France, Belgium, the Netherlands, Denmark, southern Sweden, southern Norway, Italy including Sicily, Switzerland, western Austria, western Slovenia, most of western, central and northern Germany), the second one (*O. barnabita* s.l.), including taxa distributed in central and eastern Europe (Fig. 2: most of Germany, eastern Austria, Slovenia, Czech Republic, Poland, Hungary, Romania, Bulgaria, Ukraine, Belarus, Latvia, Lithuania, Estonia, southern Finland, European Russia, Slovakia, Croatia, Bosnia and Herzegovina, Albania, Serbia, Montenegro, Macedonia, Greece, European Turkey) (Sparacio 2000; Gusakov 2002; Ranius et al. 2005; Tauzin 2006; Dutto unpublished data).
- Within the *O. eremita* s.l. clade, the taxonomic rank to be attributed to *O. italicum* (southern Italy) and to *O. cristinae* (northern Sicily) is still questionable, both taxa probably being at the border line between recently separated allopatric species and semi-species. However, at least for the geographically (Fig. 2) and morphologically more isolated *O. cristinae* from Sicily, its specific rank appears to be sufficiently supported also by our molecular data.
- Within the *O. barnabita* s.l. clade, the taxonomic rank to be attributed to *O. lassallei* seems to be less questionable owing to the evidence of nearly parapatric occurrence of both *O. barnabita* s.s. and O. *lassallei* in northwest and northeast Greece, respectively (Fig. 2), with relatively high degree of genetic differentiation.

Our study shows that there is clear evidence of genetically divergent European species and semi-species within the *O. eremita* complex, despite the fact that, in this group, the establishment of genetic thresholds delimiting taxa of specific or infraspecific rank is not always unambiguously supported by the available molecular data. However, it is rather high the risk that at least part of this genetic diversity could be eroded in the next future, due to habitat loss and fragmentation, and these preliminary data may be key to the conservation of the hermit beetles in Europe (Ryder 1986; Moritz 1995; Audisio et al. 2007).

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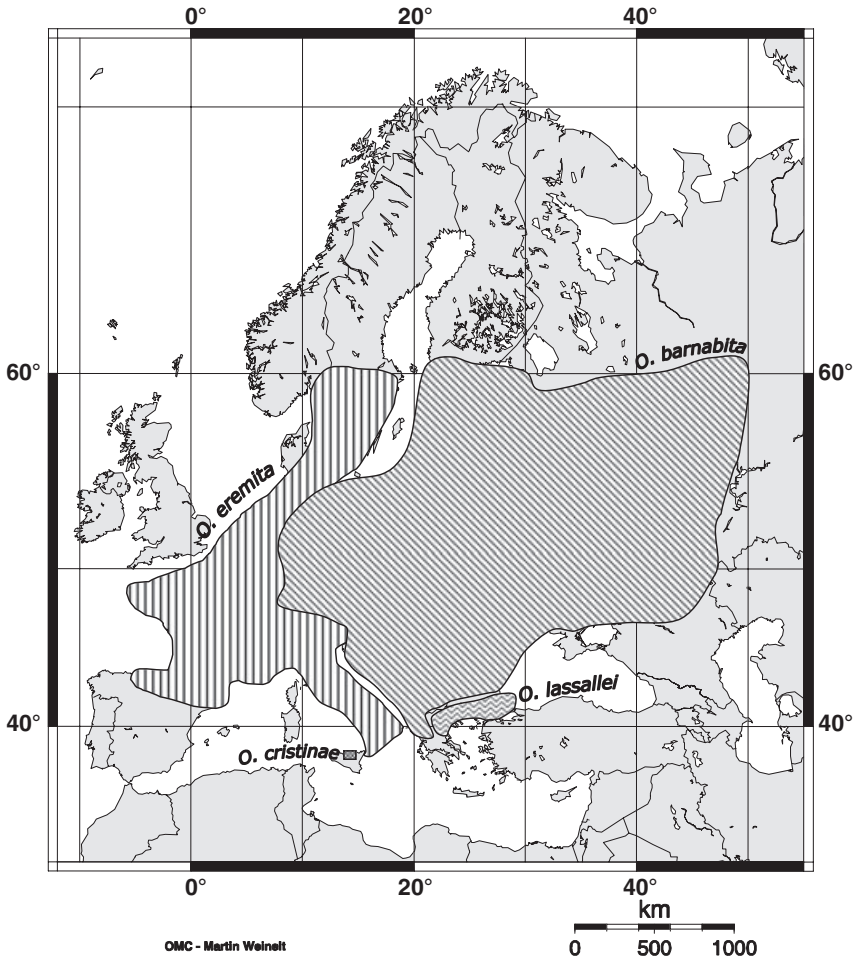

Fig. 2. Geographical ranges of the *Osmoderma eremita* complex in Europe

Mazziotta and Fabio Nicolai (Italy) for help in collecting some of the Italian specimens.

Zusammenfassung

Daten über molekulare Taxonomie und genetische Dieversifizierung der europäischen Eremiten, ein Artkomplex gefährdeter Insekten (Coleoptera: Scarabaeidae, Cetomiinae, Osmoderma*)*

An den europäischen Eremiten (oder Juchtenkäfer; *Osmoderma eremita* Art-Komplex) wurde eine molekulare Analyse zur Erforschung ihrer genetischen Diversifikation sowie der Stabilität früherer morphologisch basierender taxonomischer Anordnungen durchgeführt. Von 26 Individuen wurden vollständige Sequenzen der mtDNA des Cytochrom C Oxidase I Gens untersucht. Die Durchschnittswerte der interspezifischen Sequenzunterschiede reichen von 0.044 bis 0.186. Die Ergebnisse zeigen einen klaren Unterschied zwischen zwei Ästen. Der erste Ast beinhaltet den westeuropäischen *O. eremita* Scopoli, 1763 sowie die zwei in Italien endemisch vorkommenden *O. italicum* Sparacio, 2000; und *O. cristinae* Sparacio, 1994 von der südlichen italienischen Halbinsel, beziehungsweise von Sizilien. Der zweite Ast hingegen beinhaltet die weit verbreiteten osteuropäischen *O. barnabita* und *O. lassallei* Baraud and Tauzin, 1991 des südlichen Balkans (Griechenland und europäische Türkei). Innerhalb beider Äste stützen die molekularen und morphologischen Daten, dass es sich bei *O. lassallei* und *O. barnabita* einerseits und *O. eremita* und *O. cristinae* andererseits um eigene Arten handelt, während die taxonomische Stellung von *O. italicum*, enger verwandt mit *O. eremita*, weiter unsicher ist. Die gegenwärtige geographische Verbreitung, die interspezifische genetische Diversität und die sehr geringen intraspezifischen genetischen Unterschiede in den westeuropäischen Populationen von *O. eremita* (im engeren Sinne) lassen sich auf mehrfache Artbildungsereignisse zurückführen, die hauptsächlich in den Waldrückzugsgebieten der italienischen Halbinsel, des Balkans und Siziliens vor und während des Eiszeithöhepunkts im Pleistozän stattgefunden haben könnten. Diesen Artbildungsereignissen folgten vermutlich schnelle nacheiszeitliche Nord- und Westausbreitungen einiger Arten.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Pairwise divergences among individuals.

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